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13. ABSTRACT (Maximum 200 words) The purpose of the research was to develop reliable procedures whereby the interaction between psychological "stress" and immune function could be studied, and to begin to explore the mechanisms induced. Standard in vitro measures such as mitogen stimulated lymphocyte proliferation and NK cytotoxicity were not altered by stressors in the range that we explored in a repeatable manner. However, in vivo antibody (IgM and IgG) levels to antigen (KLH) were reliably and repeatably reduced by stressors administered soon after antigen. The stressors explored were inescapable tailshock and defeat in territorial aggression. Importantly, time spent in submissive postures rather than attack or bites predicted the reduction of antibody to KLH. Parameters such as the amount of stressor exposure and timing between stressor and antigen were explored. There are a number of mechanisms by which antibody production could have been reduced. We pursued the possibility that the stressors reduced antibody by producing a change in cell trafficking patterns such that there was an alteration of relative cell populations in some critical compartment of the immune system. We determined that the stressors used shifted CD4+/CD8+ ratios in mesenteric lymph nodes, but not elsewhere. We further obtained evidence that this change was a key factor in reducing antibody production.

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Our research in the area of stress and immunity began in 1983 (Laudenslager et al. 1983) with an experiment in which we compared the impact of exposure to escapable electric shocks (ES) and physically identical inescapable shocks (IS) on mitogen stimulated lymphocyte proliferation. Rats were exposed to 80 1.0 mA tailshocks using an escape-yoked paradigm. A typical procedure in our laboratory involves giving one group of rats a series of electric shocks delivered to the rat's tail through fixed electrodes, each of which can be terminated by performing an instrumental response such as turning a small wheel located at the front of the chamber. The shocks cannot be prevented, but each ends only when the designated response is performed. A second group of rats is given the identical series of shocks, but here the rats have no control. Each rat in this inescapable shock (IS) group is yoked to a member of the first escapable shock (ES) group. Each shock begins at the same time for both members of the pair and terminates for both rats whenever the subject who has control responds. Turning the wheel has no consequence for the second "yoked" rat. Thus both rats receive the identical durations and distributions of shock, but one has behavioral control over the termination of each shock, while the other does not. A third group is merely restrained in the apparatus. This "triadic design" allows the separation of the effects of exposure to shock per se (a comparison of escape and yoked groups with the restrained control) from the effects of control over shock (a comparison of escape and yoked groups).

Blood samples were then taken, lymphocytes separated, stimulated with the T-cell mitogens PHA and Con A, and proliferation measured by a standard [^{3}H] thymidine incorporation assay. The results were dramatic. There was no lymphocytopenia but a large (60 %) reduction in the proliferative response at the peak mitogen concentration in subjects that had experienced IS, but no reduction at all in ES or restrained subjects. Thus shock did interfere with lymphocyte function, but only if the shock was inescapable. We followed this with a similar study examining NK cytotoxicity using a ^{51}Cr release assay, and the results were congruent with those obtained for lymphocyte proliferation.

After the publication of these experiments we attempted to study the mechanism(s) responsible for these changes. However, we found the effects to be too unstable to form the basis for analytic experiments. We varied a number of parameters of the shock situation in an effort to produce a more robust and repeatable change. We manipulated shock intensity, number of shocks, number of days of shock, when the blood was obtained relative to shock exposure, the strain of rat, type of anaesthetic, colony conditions (pathogen free versus "normal"), culture conditions, and source of the lymphocytes (peripheral blood vs. spleen). None of these manipulations produced an effect that was robust enough to analyze behaviorally or physiologically. This is not to suggest that stressors cannot reliably alter the measures in question, but only that stressor parameters in the range that we have utilized do not do so reliably. Indeed, data recently reported by Weiss et al. (1989) suggests that changes in NK cytotoxicity and proliferation of lymphocytes taken from blood and spleen are most easily produced by either

very small or very large amounts of shock. These measures were reduced in rats given either 3 brief mild footshocks or 19 hours of intense shock (one shock per minute) while restrained, with little effect occurring with intermediate amounts of shock. In our experiments we did not sample shock regimens in the range of those reported to be effective by Weiss et al. We did not investigate very small amounts of shock because the behavioral processes which we wished to study such as stressor controllability effects, defeat, learned helplessness, etc., cannot be produced by a very limited exposure to the stressor such as 3 shocks. For example, 80-100 inescapable shocks are required to produce behavioral learned helplessness effects. We also did not explore the impact of very extended exposures to shock such as a 19 hr treatment. These extreme conditions produce sleep deprivation, food and water deprivation, weight loss (roughly 15 % of body weight), the production of imbalances in a variety of metabolites and metabolic processes, body temperature shifts, etc. Many of these can alter immune function directly. These sorts of conditions make it difficult to isolate the psychological aspect of stressor situations.

This state of affairs led us to rethink our approach. We and others had concentrated on in vitro measures of immune function with a primary focus on the proliferative response of lymphocytes to mitogens. However, there seemed to be a number of potential drawbacks to these measures. The major one was that it is difficult to make inferences about the overall in vivo functioning of the immune system from these measures. The immune system is composed of a complex cascade of interacting cells and processes. Thus it is quite possible that a small change in an in vitro measure as is typically observed in the stress studies, will have little relevance to the overall immune response. An experimental manipulation might depress, for example, T-cell proliferation in response to a mitogen such as PHA by 25-50 %, but the in vivo recognition and destruction of an antigen (Ag) might remain intact. The functions measured by many in vitro tests do not reflect the natural end stage of the immune response--host defense.

These factors led us to use an in vivo measure which might more faithfully reflect a natural end-product of the integrated action of the immune system. Our hope was that such a measure might be more meaningful and more reliably related to our stress conditions. We chose to examine the in vivo generation of specific Ab to an Ag. Ab production reflects many steps in the immune response from the initial processing of the Ag by accessory cells to the ultimate production of immunoglobulin by the B cells. The T-cell dependent Ag, keyhole limpet hemocyanin (KLH), was selected because it is a foreign protein to which rats have not previously been exposed, and while harmless, is highly immunogenic. We modified an enzyme-linked immunosorbent assay (ELISA) for KLH IgG Ab. Our pilot investigations confirmed that rats begin with no detectable Ab to KLH and develop Ab to KLH in a regular and dose-dependent fashion.

In our first set of experiments rats (Laudenslager et al. 1989) (N=12 per group) received either 1 or 3 daily sessions of IS per week on two successive

weeks. One hundred 5-s inescapable tailshocks were delivered, with shock intensity increasing every 25 shocks from an initial value of 0.8 mA to a final value of 1.6 mA. We chose this procedure because it maximizes some of the behavioral effects of IS. Control animals were maintained in their home cages but brought to the laboratory environment during shock sessions. That is, they were removed from the animal colony and transported to the same area of the laboratory in which the experimental animals were treated. This was done to equalize movement, handling, and exposure to any pathogens which might have been present in the experimental environment. IS and ES were not compared in these intitial studies because we wished to establish a repeatable effect of our shock conditions before exploring factors such as stressor controllability which require a much larger number of subjects.

Prior to the first session of shock each week, all animals were immunized with a subcutaneous injection of 0.2 ml KLH suspension (5 mg/ml sterile saline). Exactly 8 weeks following the first immunization a tertiary response was evoked by a third injection of KLH. Because of our concern that any effect might be sensitive to circadian influences and because we wished to establish the generality of any effects obtained, half of the animals were immunized and shocked during the light part of their 12:12 day/night cycle, and half during the dark part. Blood samples were obtained from the tail vein using a standard procedure in our laboratory that requires very little disturbance of the rat. This was done 1, 2, 3, 8, 9, and 10 weeks following the first immunization with KLH.

The results for the 4 conditions and controls for each are shown in Fig. 1. The data presented here are KLH IgG Ab levels (absorption units) for a 1:1000 serum dilution. Comparisons are never made across "runs" of the assay. The response to KLH increased across the first 3 weeks and declined by Week 9. Each of the 4 shock conditions (1 and 3 sessions occurring in the day or night) reduced the development of Ab to KLH to a roughly equal degree. This effect increased as Ab level rose. Recall that the last immunization and shock sessions occurred during Week 2. Thus the difference in Ab levels at Week 4 reflects a difference remaining 2 weeks after the last experimental treatment. Moreover, the impact of IS was still evident 7 weeks after the last shock experience. Further, these differences remained reliable at <u>all</u> dilutions from 1:200 to 1:12,800, beyond which the assay cannot detect Ab.

Recall that we also wished to explore the impact of defeat in aggressive encounters. To this end we developed a colony-intruder paradigm in our laboratory as a model of territorial aggression. In this paradigm 2 males are allowed to live in the same environment (a large tub cage) for several months. One of the males will become dominant during that time. An "intruder" is then introduced. The dominant male will attack the intruder with a fairly short latency and the intruder is removed after 10 min. During this period attack will usually have occurred and it is ordinarily enough to induce submissive posturing in the intruder. The intruder initially retaliates, then engages in defensive reactions, such as the defensive upright posture and escape attempts. Eventually the intruder displays a submissive response

pattern characterized by upright body posture with raised limp forepaws, upwardly angled head, and retracted ears. Frequently the animal will turn over on its back and expose its ventral surface. Once defeated, rats show these behavioral features even before being attacked and fail to orient toward the opponent. The intruder can thus be returned for any number of sessions while producing little if any bites or physical injury. Many details of the procedure are crucial to producing reliable aggression in the resident and defeat in the intruder (e.g., resident males must be quite large, sessions must be conducted during the dark part of the resident's cycle, colony diads must have lived together for several months and have experienced previous intruders since colonies become more aggressive with experience, etc.). We have now had sufficient experience with this procedure so that we can produce very reliable aggression and defeat of the intruder. With our procedures the intruder invariably "gives up", probably because it "knows" it is in another male's terrritory via odor cues. We videotape the encounters and have developed a very reliable scoring procedure (we count number of bites, latency to adopting a defeat posture, amount of time spent in submissive postures, etc.) with inter-rater reliabilities of over .93.

Our first experiment was modeled after the shock study (Fleshner et al. 1989). Rats (N=12) were immunized with KLH on Days 1 and 8 and exposed to 50 min of defeat experience after each of the immunizations. Fifty min of defeat was arranged by successively exposing the rats to 5 different aggressive colonies, each for 10 min. Simply allowing an intruder to remain in a single colony for 50 min will not produce sustained submissive posturing or aggression. Control rats (N=12) were exposed to successive nonaggressive colonies for an equal period of time. Thus these animals were moved, placed in the presence of unfamiliar conspecifics, etc, but were not attacked and therefore did not submit. Blood samples were taken on Weeks 2, 3, and 4 and serum IgG Ab to KLH determined.

The introduction of the intruder into resident colonies depressed Ab measured several weeks later. This defeat procedure has the advantage over the IS procedure in that a variety of behavioral measures result (e.g., bites, submission latency, total submission time during the 50 min, etc.). This allows one to determine whether any predict the magnitude of Ab level. We examined simple correlations, partial correlations, and performed stepwise multiple regressions using a large number of predictor variables. The interesting result of each of these ways of examining the data was that bites did not predict Ab level ($r = .11$). Indeed, there were cases of rats which did not receive a single bite or even physical contact, but showed low Ab levels. By far the best predictor was time spent in submissive postures ($r = .68$). Indeed the r^2 between submission time and Ab level, partialling out bites, was .71.

Refinements of Procedure

1. The experiments described above employed 2 injections of lyophilized KLH separated by 1 week, with the stressor applied after each injection. A double injection-stressor procedure makes "timing" studies and

neuroendocrine manipulations difficult. Lypohilized KLH also has disadvatages in that it may not "clear" rapidly. We have therefore explored the use of a single administration of soluble KLH. We began by examining a variety of doses in order to find a single dose which produces a robust Ab response, but that is at the lower end of the effective range so that the system is not "overwhelmed" by KLH. A dose of 100 ug was selected and a single session of IS delivered following this dose of KLH, suppressed the formation of anti-KLH IgG. Data are in Fig.2. It was desirable to measure anti-KLH IgM as well as IgG since the initial Ab response to an Ag is almost exclusively IgM. We modified our ELISA in order to enhance our ability to detect low levels of IgM Ab. We then examined the effects of IS, and it suppressed IgM to KLH as well as IgG . Data are shown in Fig.2.

3. We reasoned that it would be useful to measure the number of Ab secreting cells (ASCs), in addition to Ab levels. The most common assay for this purpose is the hemolytic plaque assay. However, this assay has a number of disadvantages that have frequently been noted. Highly sensitive enzyme-linked immunospot assays (ELISPOT) have recently been developed to measure ASCs, and these procedures offer many advantages. These assays can be thought of as similar to ELISAs in that the tests measure the amount of anti-KLH Ab (either IgG or IgM dependent on the secondary Ab used) produced by the rats. One critical difference between the ELISA and the ELISPOT is that the anti-KLH Abs for the ELISA are circulating Abs contained in serum samples whereas the anti-KLH Abs in the ELISPOT are secreted during the assay from isolated B-cells (from spleen or mesenteric lymph nodes) in direct response to the KLH which coats the microtiter wells. The second critical difference between the ELISA and the ELISPOT is the endpoint of the assay. The ELISA uses a soluble yellow reaction product and the total amount of that reaction product in each well is quantified (in terms of absorption units). In contrast, the ELISPOT uses a non-diffusable blue reaction product which forms only at the spots on the microtiter well contacted by each KLH-specific ASC. The total number of these highly discrete blue spots in each well represents the number of ASCs contained in the well. We have therefore established an ELISPOT assay in our laboratory to detect Ab cells secreting KLH-specific IgG and IgM separately. We have varied a number of aspects of this assay to optimize results and have put substantial effort into it. Some of these efforts have been (a) determining the best method of isolating lymphocytes from spleen and mesenteric nodes (Ficol separation proved superior to a variety of lysing buffers) (b) determination of the optimal concentration of agar in the substrate mixture to minimize endproduct diffusion while maintaining ease of substrate application and maximizing detection of reaction product by maximizing the clarity of the agar; (c) determining the optimal volumes and optimal aliquoting techniques for the cell suspensions and agar-substrate mixtures that are pipetted into the plates in order to attain highly replicable, even, thin coverage. For cell suspensions, dispersal in an even, thin layer results in evenly distributed, well-defined blue spots which are reliably counted. For

agar-substrate mixtures, dispersal in an even, thin layer optimizes the ease of counting the spots that form; (d) determining the optimal procedures for coating the microtiter plates with Ag to maximize reliability and sensitivity; and (e) determining the timecourse of ASC development in response to ip soluble KLH in both mesenteric lymph nodes and spleen. With the modifications that we have made the assay is now operating quite well.

We have used the ELISPOT to examine the timecourse of the formation of ASCs for both IgM and IgG following immunization with KLH. Rats were immunized with 100 ug soluble KLH ip and spleens removed 5, 7, or 9 days later. Results can be seen in Fig. 3. The peak in the number of primary (IgM) plasma cells was on Day 7 post-immunization. The largest number of secondary (IgG) plasma secreting cells was on Day 9. We are currently exploring other timepoints to determine whether the peak IgM might not be on Day 6 (or 8), and whether IgG cells might not continue to increase after Day 9. The rest of this proposal will assume that thpeak IgM is on Day 7. If it turns out to be on Day 6 or 8 the experiments will be adjusted accordingly. We are also beginning to use this assay to evaluate whether the IS procedure alters the number of ASCs.

4. A major criticism that several individuals have made is that the stressor might be reducing protein "in general", rather than influencing Ab specific to KLH. To address this point, we have collected tail vein serum samples from 12 rats that were <u>not</u> immunized with KLH (6 home cage controls; 6 shocked using our normal protocol) immediately post-shock, and 1, 5, and 14 days post-shock. 100 ul aliquots of these samples were applied to Isolab System II ion exchange columns in order to collect fractions enriched with total IgG and fractions enriched with total IgM. These total IgG and total IgM fractions were then assayed for total protein content (Bradford protein assay). The results can be seen in Figure 4 and providfe no support to the protein reduction argument.

5**. It is sometimes convenient to group house animals in these experiments, and other times to individually house them. We try to keep the animals in our immunology research individually housed, but there are occassions when space demands require group housing. We thus conducted our standard IS experiment with individually housed animals, group housed animals,(3 rats to a standard size group tub), and animals that had been switched from either group to individual or individual to group 24 hr earlier. Housing conditions had <u>no impact</u> on the magnitude of the IS effect on Ab level.

6. We tested a number of details of our <u>in vitro</u> protocol to determine their impact, and made some improvements. (a.) We have compared the effects of various tissue collection methods (n=6-8 rats/condition) on proliferative (PHA and Con A) responses of peripheral blood lymphocytes and splenocytes. Splenocytes collected under pentobarbital anesthesia showed suppressed proliferation, compared to cells collected under (i.) brief ether followed by cervical dislocation or (ii.) decapitation. Peripheral blood lymphocytes likewise were suppressed when collected under pentobarbital anesthesia

compared to either (i.) brief ether followed by cervical dislocation or (ii.) collection from unanesthetized animals pre-implanted with indwelling internal carotid cathethers. In both cases, tissue collected under brief ether and from unanesthetized rats (decapitation or indwelling cathether) showed equivalent proliferative responses. (b.) Our in vitro methods were developed to test proliferative responses of isolated (washed) peripheral blood lymphocytes and isolated splenocytes. We wished to modify these methods to allow us to test these cells in the presence of all cells, hormones, etc. present in the original tissue. We have successfully modified our procedures to allow us to optimally test the proliferative responses of lymphocytes in whole blood and whole spleen cell suspensions; in addition, we can also now optimally test lymphocytes from cervical lymph nodes and mesenteric lymph nodes as well. For each tissue type, these modifications have involved systematic determinations of optimal cell concentrations per well, peak mitogen concentrations (PHA, ConA), optimal incubation times and conditions, etc.

Parametric Information

1. Number of inescapable shocks. Separate groups (N=10) were immunized with 100 ug soluble KLH and then given either 0, 5, 10, 20, 40, 60, 80 or 100 inescapable 5-sec 1.6 mA tail-shocks while restrained according to our usual procedures (see App. 4). Blood samples were taken 3, 5, 7, 9, 14, and 21 days later. In contrast to the in vitro results reported by Weiss et al. (1989), small amounts of shock had no effect whatsoever. Only the 80 and 100 shock conditions altered anti-KLH IgM and IgG, with the effect being more robust after 100 shocks.
2. Duration of shock session. We reasoned that distributing the 100 shocks out over a longer time period might produce an even larger effect than we had observed with our usual procedure. In our typical procedure a shock occurs with an average inter-shock interval of 60 sec. Here we gave separate groups 100 shocks with inter-shock intervals of either 60, 120, or 180 sec. The longer session durations did not magnify the changes in Ab to KLH.
3. Time between immunization and shock. Zalcman et al. (1989) reported that shock given to mice 72 hr after immunization with SRBC reduced the plaque forming cell response, but that shock given immediately after immunization either had no effect or actually enhanced the Ab response. This is a suggestive finding since 72 hr after immunization should be near the point of maximum clone formation. We thus undertook a large timing study. Separate groups of rats (N=12 per group) were given our typical shock procedure either 96, 72, 48, or 24 hr before immunization with 100 ug KLH, immediately after KLH, or 24, 48, 72, or 96 hr after KLH. Blood samples were taken at the usual timepoints after immunization, and anti-KLH IgG and IgM assessed. Shocks given 96, 72, and 48 hr before immunization and 24, 48, 72, or 96 hr after immunization clearly had no effect. As usual, shock immediately after immunization suppressed Ab levels. Interestingly, shock given 24 hr before immunization suppressed Ab levels, although it should be cautioned that we need to replicate this finding (study now underway).

Clearly, these timing relationships are quite different than those reported by Zalcman et al. with a different Ag, species, and stressor.

Cell Subsetting and Trafficking

There are many potential mechanisms which might produce stress-induced immunomodulation. The earliest hypotheses focused on cell death or cell dysfunction. A more recent proposal is that stress alters cell trafficking patterns of different cell types between compartments of the immune system. Since most immune responses require the cooperation of many different cell types, shifts in specific cell populations in some critical compartment could render an organism less able to mount a normal immune response to antigenic challenge to that compartment. Here each individual cell type functions normally, it is their ability to cooperate normally that is impaired because their balance in relationship to each other is altered.

With this possibility in mind and to more fully characterize the immune changes produced by the stress protocol which we have found to alter in vivo Ab levels to KLH, we examined the impact of this stress protocol on lymphocyte populations in different compartments. Lymphocytes from peripheral blood, spleen, cervical lymph nodes, and mesenteric gut lymph nodes were collected immediately after IS or control treatment. These compartments were chosen because they include the major tissue types which are involved with lymphatic migration and humoral immune responses. In collaboration with Dr. D. Bellgrau, T-cell subsets and B-cells were counted by flow cytometry using fluorescein isothiocyanate labeled W3/25, Ox8, Ox12, and Ox19 Abs. This labeling procedure identified cells positive for the cell surface markers CD4, CD8, Ig, and CD5, respectively. CD5 is present on all T-cells. CD4 and CD8 are markers present on T-cells specific for class II and class I Ag respectively, coded for by the major histocompatibility complex. These markers were chosen because they identify discrete sets of lymphocytes which are principally involved with mounting an Ab response. CD4+ cells tend to be helper/inducer and CD8 suppressor/cytotoxic, but there are exceptions (see below). The flow cytometric data was analyzed in terms of the percent of total cells sampled that were positive for the marker, minus percent nonspecific binding. Monocytes were excluded from analysis using 90^{o} scatter.

Shock did not induce measurable shifts in cell populations in peripheral blood, spleen, or cervical lymph nodes. However, there were large shifts in the mesenteric lymph nodes. The percentage of CD4+ cells reliably increased (Fig. 5) and the percentage of CD8+ cells decreased (Fig. 6) after shock. This resulted in a very large increase in the CD4/CD8 ratio in the mesenteric nodes produced by shock (Fig. 7). The increase in CD4+ was larger than the decrease in CD8+, and this was reflected in an increase in the percentage of CD5+ cells produced by shock (Fig. 8), supporting the view that the CD4+ and CD8+ cells were indeed T-cells. There were no changes in Ig+ cells (Fig. 9).

In our next experiment we sought to determine if this shift in CD4+ and CD8+ cells in the mesenteric nodes might be critical in producing shock-induced suppression of Ab to KLH. We reasoned that if KLH were administered in such a way that it would be processed in compartments other

than the mesenteric nodes, then Ab formation should be unaffected by shock. However, if the KLH were administered in such a way that it would be at least partially processed in the mesenteric nodes, then shock ought to interfere with Ab levels. Ag administered intravenously (iv) is processed primarily by the spleen, whereas Ag administered intraperitoneally (ip) is processed by both spleen and mesenteric nodes (Tizard, 1984). Thus we compared the effects of our typical IS procedure on Ab formation to KLH after either iv or ip administration. As expected, IS decreased anti-KLH IgM and IgG after ip administration. The new finding was that shock was completely without effect on Ab formation after iv administration. Of course, iv and ip administration produce different overall levels of Ab to KLH, and dispersal after an ip administration is slower than after iv. Thus we employed a number of different doses of KLH administered iv, and explored a number of different temporal intervals between iv administration and shock. In no case did shock alter Ab, either IgM or IgG, after iv administration!

This encouraged us to further explore the CD4/CD8 change in the mesenteric nodes. The larger of the two changes is the increase in CD4+ cells. Two possible explanations for this increase are that the shock might cause some population of mesenteric cells to begin expressing CD4+, or more likely, CD4 cells might be driven from another lymphoid compartment and "trapped" in the mesenteric nodes. A number of hormones and neural factors have been shown to be capable of producing the latter change (Cohen, 1972).

A probable source of CD4+ cells that could move into mesenteric nodes during shock is the spleen. We did not detect a change in percent CD4+ in spleen in the experiment above, but the number of cells in spleen is vastly greater than that in the mesenteric nodes, and the migration of a number of cells sufficient to shift percentages in the mesenteric nodes might not be observable in spleen. Thus in our next experiment we determined whether splenectomy would alter the ability of inescapable shock to shift phenotypes in the mesenteric nodes. Subjects were splenectomized or given sham surgery 2 weeks prior to shock. Shock produced the previously observed shift in subpopulations in mesenteric nodes for sham surgery animals, but did not do so in splenectomized subjects (Fig. 10).

If the CD4+/CD8+ shift in mesenteric nodes is really critical to producing the depression in Ab levels after shock, then splenectomy should also prevent the effect of shock on Ab levels. This possibility was pursued in our next experiment. Rats were splenectomized or given sham surgery 2 weeks before either iv or ip immunization with KLH followed by shock or control treatment. Blood was then sampled over the course of 21 days according to our typical protocol. As usual, IS had no effect on anti-KLH after iv administration under any condition. The important new result was that splenectomy prevented the suppressive effect of shock on Ab after ip administration (Fig. 11). This would seem to be a powerful finding. It is counterintuitive that an animal immunologically compromised by splenectomy would be resistant to the effects of stress. Moreover,

splenectomized subjects produced less Ab to KLH, as would be expected. A variety of data indicates that it is easier to suppress Ab levels at lowe · levels of Ab.

In a final experiment we addressed a potential difficulty with some of the experiments above. In the experiments that examined the effects of IS on cell trafficking the animals had not been immunized with KLH. Since Ag itself can alter trafficking patterns there is a potential confound. Thus we repeated the basic trafficking experiment in animals that were immunized with KLH. The same effects as described above were observed (Fig. 12).

Finally, mention should be made that since many of the above experiments have required sacrifice of the animals at various times after inescapable shock, we have recorded the weights of a variety of organs after control treatment, immediately after inescapable shock or 24 , 48, 72, or 96 hr after IS. We have complete data for spleen, thymus, adrenal glands, and liver. Only the spleen has shown changes, with large reductions in weight after IS which persist for at least 96 hr (Fig. 13). Since the reduction in spleen weight could reflect general fluid loss we have also monitored blood hematocrit and body weight. Hematocrit is not altered by IS and there is no body weight change right after the shock session, the point at which spleen weight is maximally reduced.

The implication of these experiments is that stress-induced modulation of in vivo Ab levels might occur in association with cell trafficking. Shock may cause a nonspecific and global movement of lymphocytes from the spleen. Interestingly, catecholamies are known to produce such an effect (Cohen, 1972). A nonspecific secretion would not produce any change in subset percentages as measured by our flow cytometry procedure. Following the release of splenic lymphocytes $CD4^+$ cells might move into and be trapped in the mesenteric nodes. This trapping could be mediated in a variety of ways and lead to $CD8^+$ cells moving out of the mesenteric lymph nodes, thus altering the immune response to Ag processed at this site. These lymphocytes are probably T-cells since shock was also associated with an increase in CD5+ cells, all T-cells. This increase was not as large as the CD4+ increase, probably due to a concurrent decrease in CD8+ which are also CD5+ cells. Importantly, the stress-induced changes were compartmentally specific. That is, mesenteric but not cervical lymph nodes were affected by stress. This finding is reasonable because these nodes have different blood-lymphatic draining routes, different high endothelial binding factors (HEBF), which are important for lymphocyte movement into lymph nodes, and different blood circulation patterns, which are related to lymphocyte recruitment .

There are several ways that IS could cause changes in cell trafficking. First, IS could cause changes in any of the factors described above (i.e., lymphatic-blood recirculation, HEBF, and blood circulation), which could result in the changes in lymphocyte recirculation and lymph node trapping. Second, IS could exert its effect via hormonal routes. High levels of both glucocorticoids and epinephrine affect lymphocyte recirculation and sequestration (Cohen, 1972), and are produced by stressors. Third, lymphocyte

recirculation could be altered by sympathetic activation. Specifically, sympathetic activation of the splenic NE fibers could be responsible for driving $CD4^+$ cells out of the spleen and into the lymphatic circulation. This idea is consistent with the findings that NE fibers are associated with $CD4^+$ and $CD8^+$ splenocytes (Barclay,1981) and that pharmacological doses of NE result in release of splenic lymphocytes and granulocytes. Fourth, both splenic sympathetic innervation and adrenal medullary hormones could be important for lymphocyte recirculation given that medullary catecholamines can be sequestered in the spleen, and could perhaps activate splenic sympathetic innervation causing lymphocyte mobilization.

Studies in progress that will soon be complete

1. Time course of cell trafficking changes. We know that there is a 24 hr window after shock during which KLH can be administered and Ab formation depressed. It is thus of interest to determine how long the shift in CD4+ and CD8+ cells in the mesenteric nodes persist. It is also possible that other compartments might show changes at different times post-shock. Thus subpopulations in blood, spleen, cervical nodes, and mesenteric nodes will be assessed at various times after shock. We have complete data at thel hr and 48 hr time points and the CD4/CD8 changes in mesenteric nodes is till present atl hr, and no other compartment shows any shock-produced shifts. There are no longer any shifts at 48 hr. Data for 6 hr and 24 hr is now being collected.
2. All of the cell trafficking studies have used IS as a stressor. We simply have not had a chance to examine defeat. To increase generality and to provide a basis for some of the studies to be proposed we are examining the impact of defeat in aggressive encounters on cell subsets in different compartments in a manner identical to that which we have conducted for IS. These data are not complete, but appear to be quite similar to the shock data.

In summary, we have found that 2 different experiences (IS and defeat) produce decreases in the in vivo levels of both IgM and IgG Ab specific to an Ag administered within 24 hr of the experience. Moreover, IS decreases the number of ASCs with regard to both IgM and IgG. We have studied IS in detail, and know some of the basic parameters determining its effect on Ab. We also have a more complete characterization of the impact of this stressor on in vivo changes in immune function and organs, and have preliminary indications concerning what sorts of changes might be key. The compartmental specificity of these changes is encouraging and suggests that our stressor is not merely producing some generalized nonspecific effect.

Figure Captions

Fig. 1. Anti-KLH IgG after 1 or 3 sessions of IS in either the light or dark phase of the cycle, or after control treatment.

Fig. 2. Anti-KLH IgG and IgM following a single immunization with 100 ug soluble KLH ip. Subjects received either IS or control treatment immediately after immunization.

Fig. 3. KLH-specific IgM and IgG plasma cells in spleen 5, 7, and 9 days after immunization with 100 ug soluble KLH ip.

Fig. 4. Serum IgM and IgG immediately, 1, 5, 9, and 14 days after IS or control treatment. IgM is at a 1:20 dilution and IgG at 1:91. An absorbance of 1.00 is calibrated at 1 mg of protein per ml.

Fig. 5. % CD4 positive cells in spleen peripheral blood, cervical nodes, and mesenteric nodes after IS or control treatment.

Fig. 6. % CD8 positive cells in spleen,peripheral blood, cervical nodes, and mesenteric nodes after IS or control treatment.

Fig. 7. %CD4+/%CD8+ in spleen, peripheral blood, cervical nodes, and mesenteric nodes after IS or control treatment.

Fig. 8. % CD5 positive cells in spleen,peripheral blood, cervical nodes, and mesenteric nodes after IS or control treatment.

Fig. 9. % Ig positive cells in spleen,peripheral blood, cervical nodes, and mesenteric nodes after IS or control treatment.

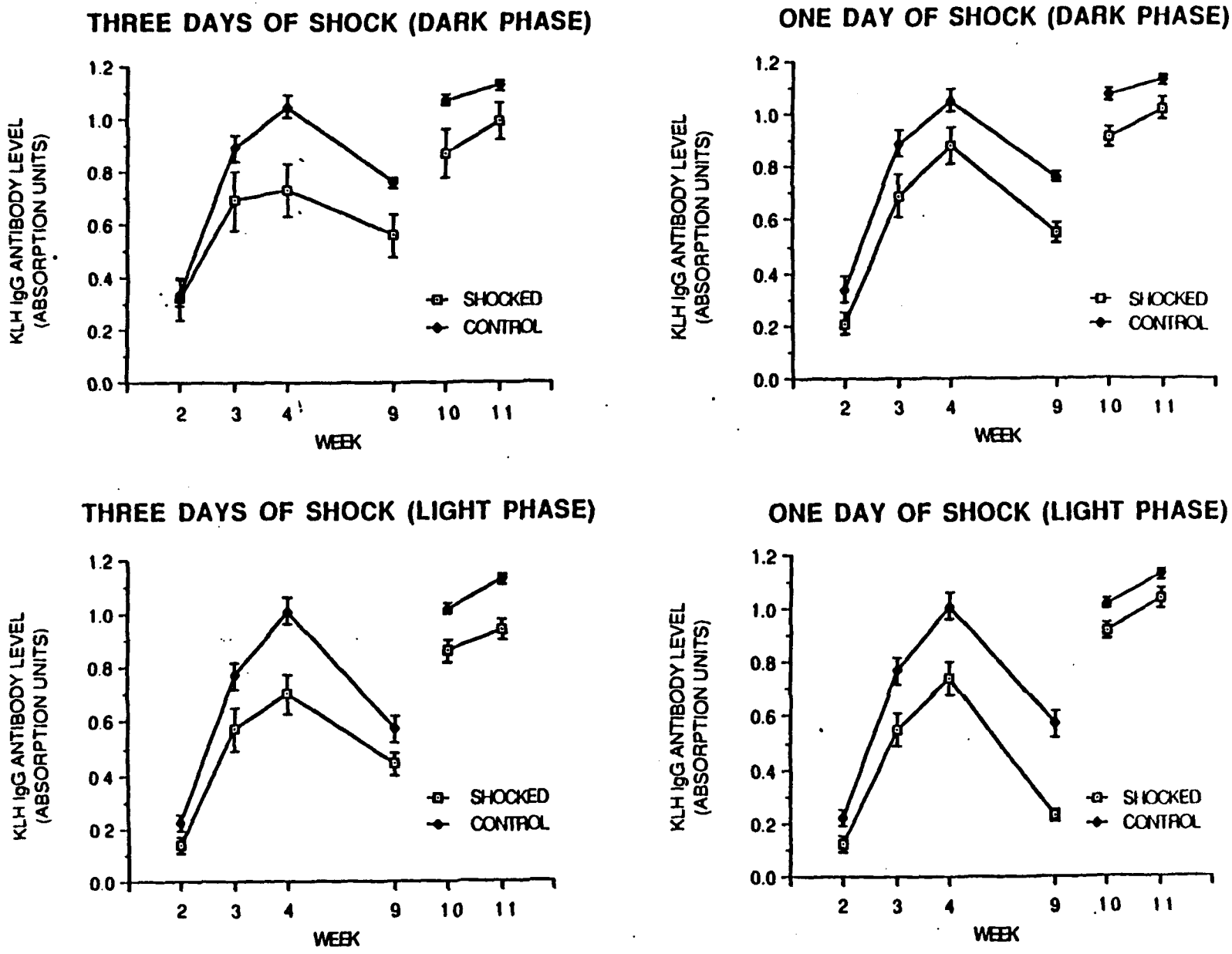
Fig. 10. %CD4+/%CD8+ from mesenteric nodes in slenectomized or sham surgery subjects. Subjects received either IS or control treatment before sacrifice.

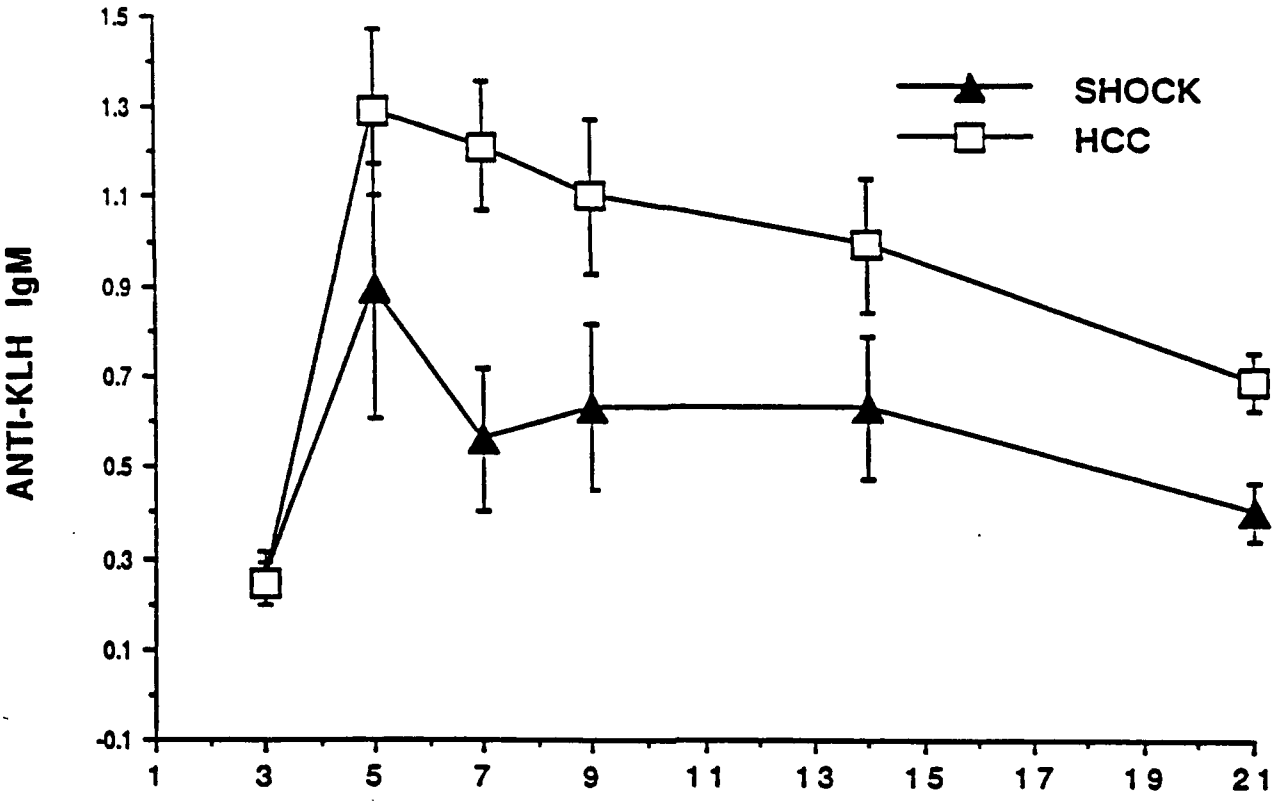
Fig. 11. Anti-KLH IgM and IgG in splenectomized animals that received either IS (SK) or control treatment before sacrifice.

Fig. 12. %CD4+/%CD8+ in spleen, cervical nodes, and mesenteric nodes after IS or control treatment.in animals that had been immunized with 100ug soluble KLH ip.

Fig. 13. Spleen weights for controls and animals sacrificed at various times after exposure to IS.

Figure 1


THREE DAYS OF SHOCK (DARK PHASE)
ONE DAY OF SHOCK (DARK PHASE)
THREE DAYS OF SHOCK (LIGHT PHASE)
ONE DAY OF SHOCK (LIGHT PHASE)
KLH IgG ANTIBODY LEVEL (ABSORPTION UNITS)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2
3
4
9
10
11
WEEK
SHOCKED
CONTROL


SHOCK-INDUCED REDUCTION OF IgM IN IP KLH IMMUNIZED RATS
ANTI-KLH IgM
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
1
3
5
7
9
11
13
15
17
19
21
SHOCK
HCC

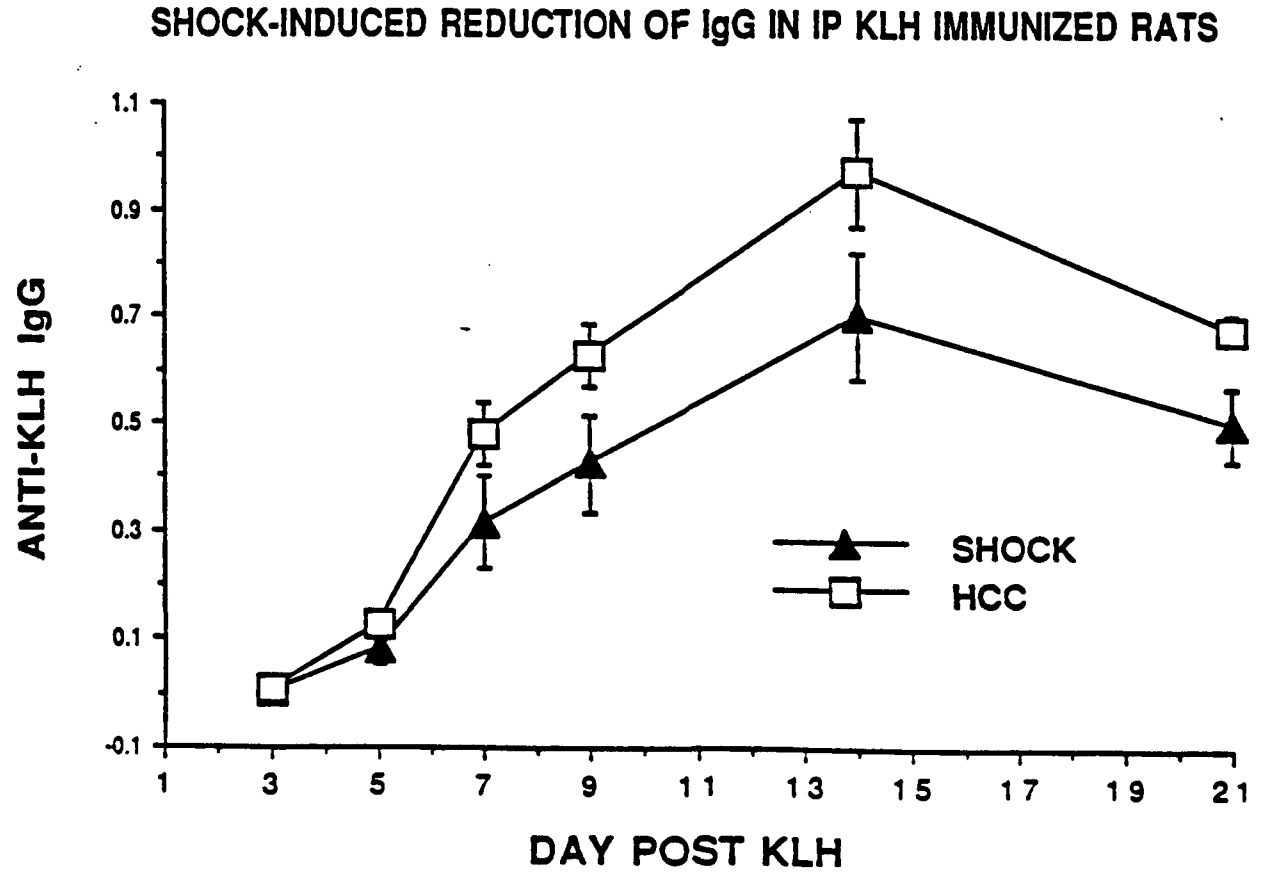
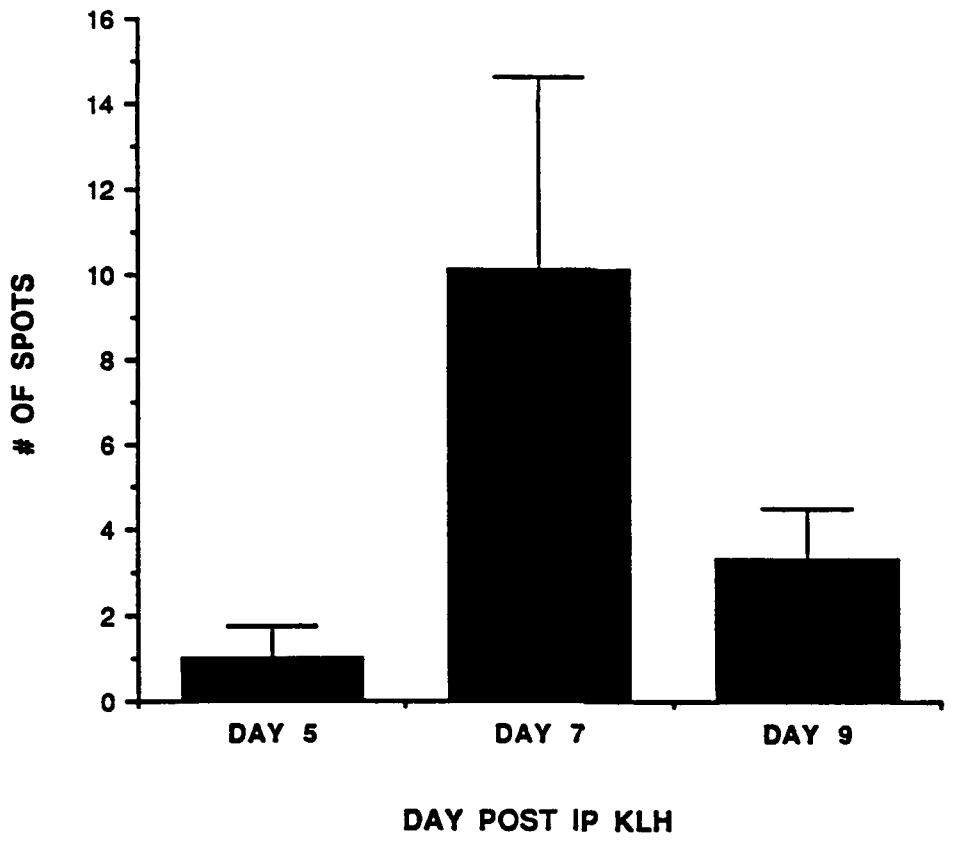

SHOCK-INDUCED REDUCTION OF IgG IN IP KLH IMMUNIZED RATS
ANTI-KLH IgG
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
1
3
5
7
9
11
13
15
17
19
21
SHOCK
HCC
DAY POST KLH

Figure 2


KLH-SPECIFIC IgM PLASMA CELLS IN SPLEEN
OF SPOTS
16
14
12
10
8
6
4
2
0
DAY 5
DAY 7
DAY 9
DAY POST IP KLH

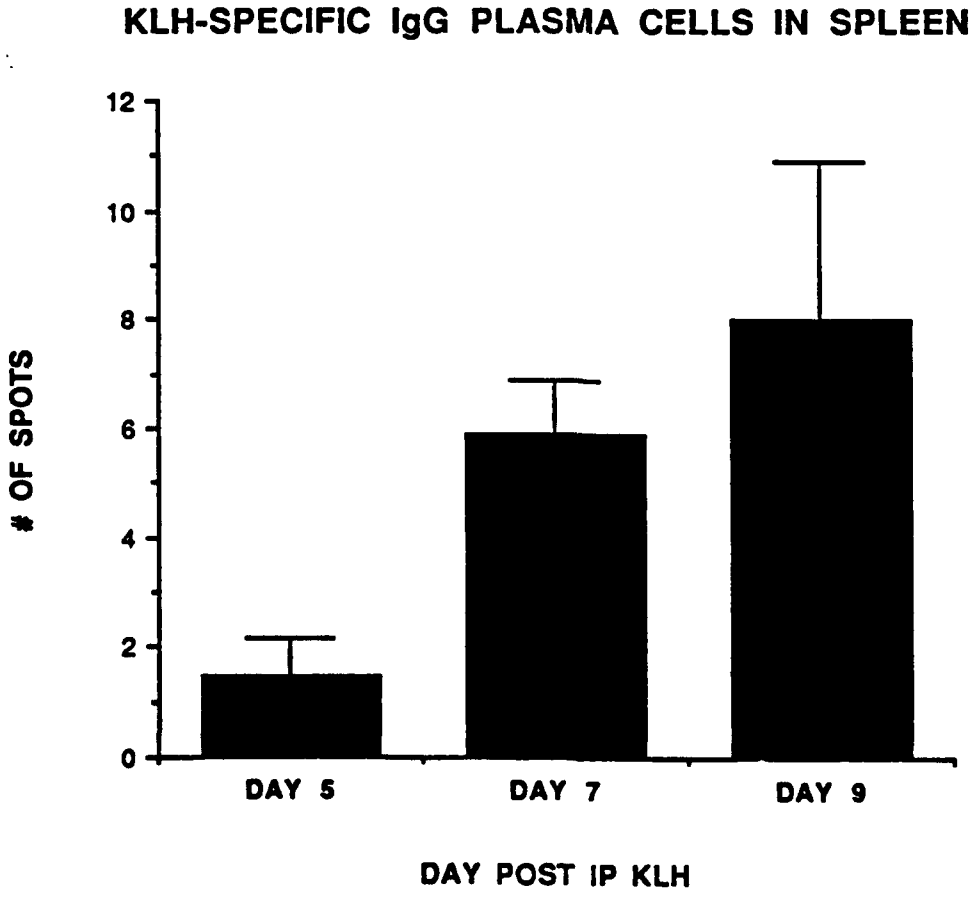
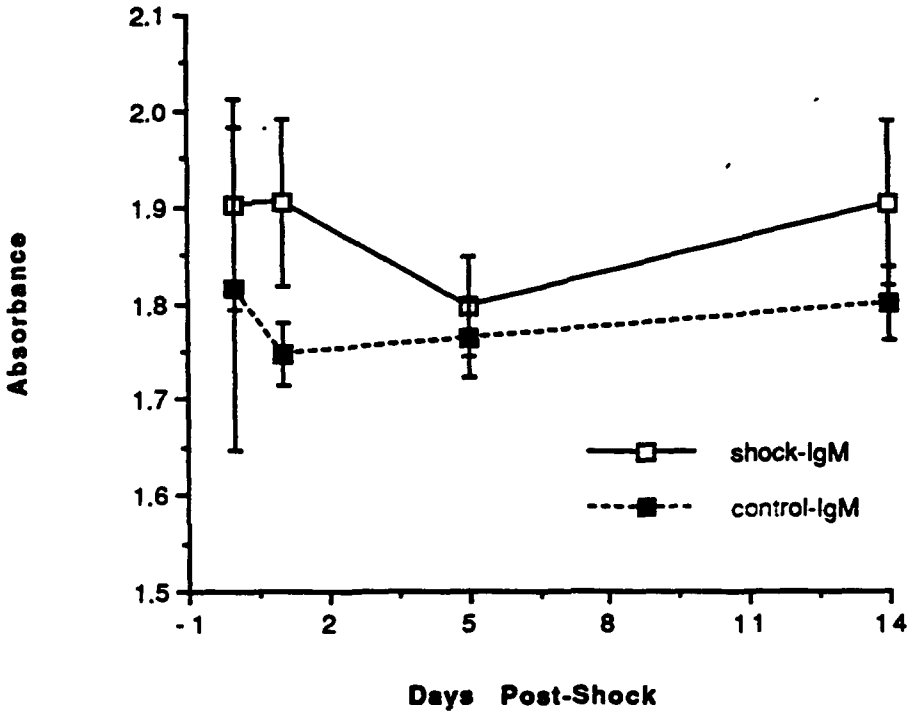

KLH-SPECIFIC IgG PLASMA CELLS IN SPLEEN
OF SPOTS
12
10
8
6
4
2
0
DAY 5
DAY 7
DAY 9
DAY POST IP KLH

Figure 3

Effect of Shock on Total Serum IgM



Effect of Shock on Total Serum IgG

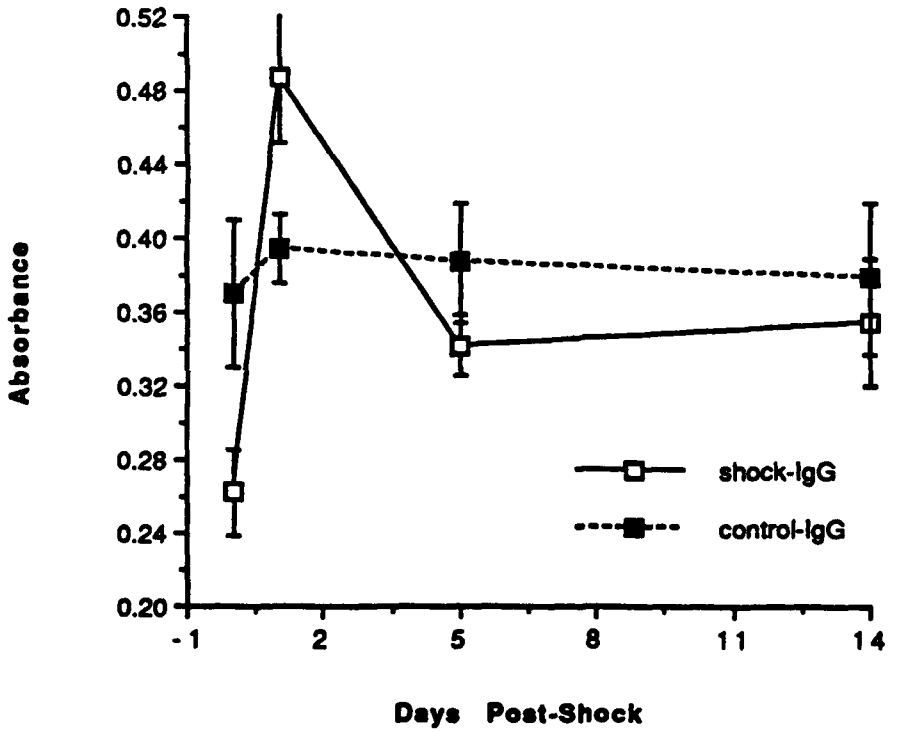


Figure 4

A SHOCK-INDUCED INCREASE IN CD4+ MESENTERIC LYMPHOCYTES

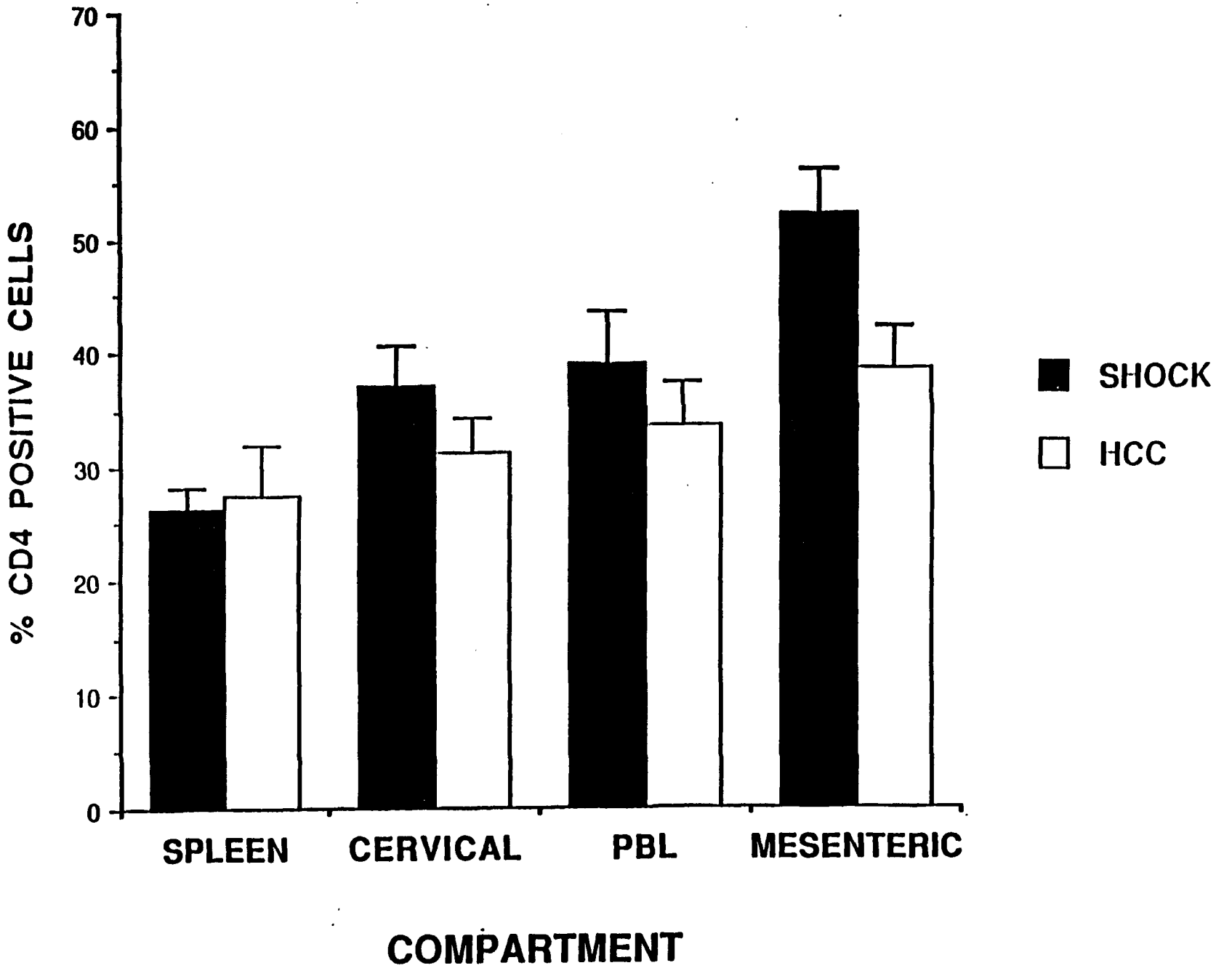


Figure 5

A SHOCK-INDUCED DECREASE OF CD8+ MESENTERIC LYMPHOCYTES

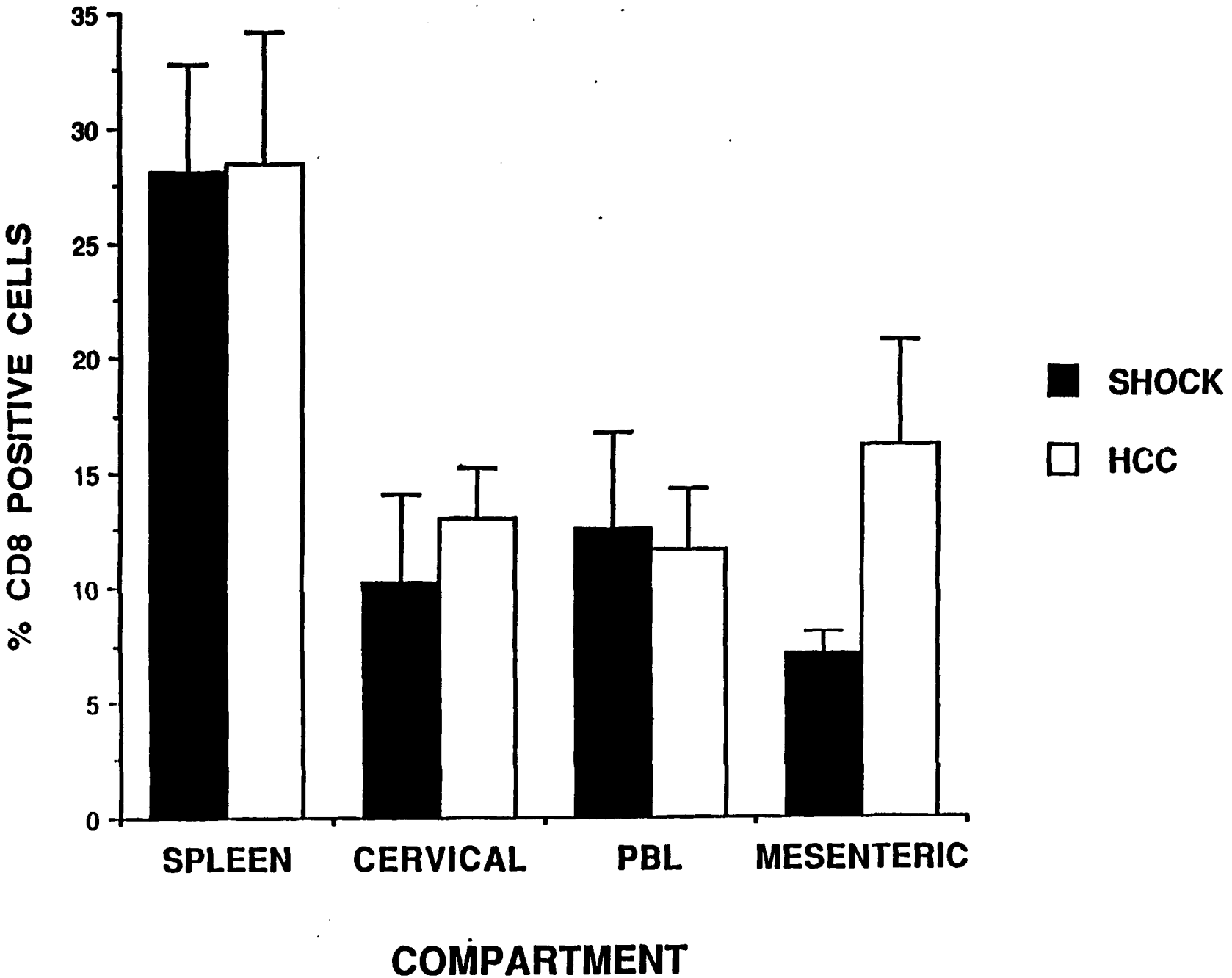


Figure 9

COMPARTMENT SPECIFIC STRESS-INDUCED INCREASE OF CD4/CD8

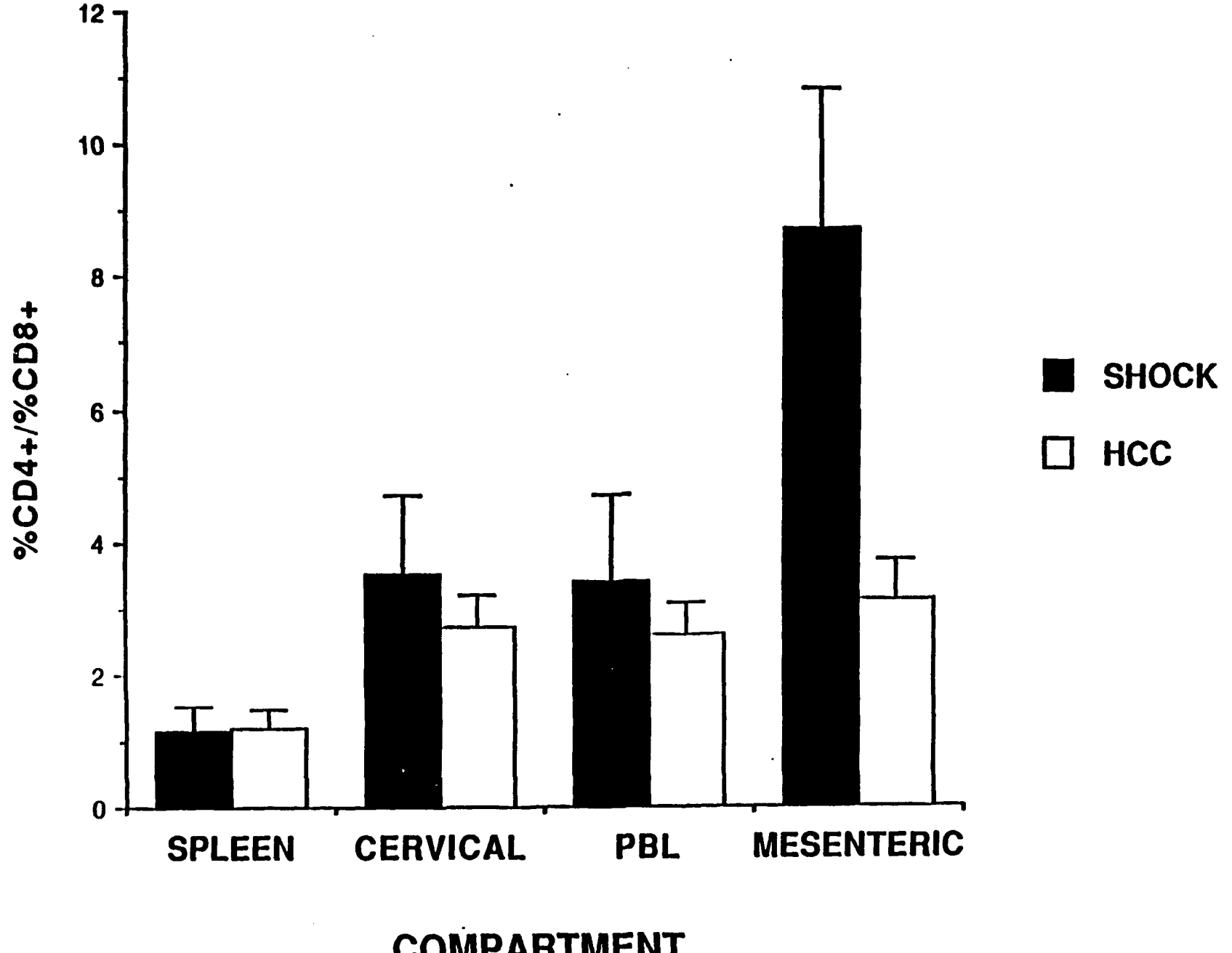
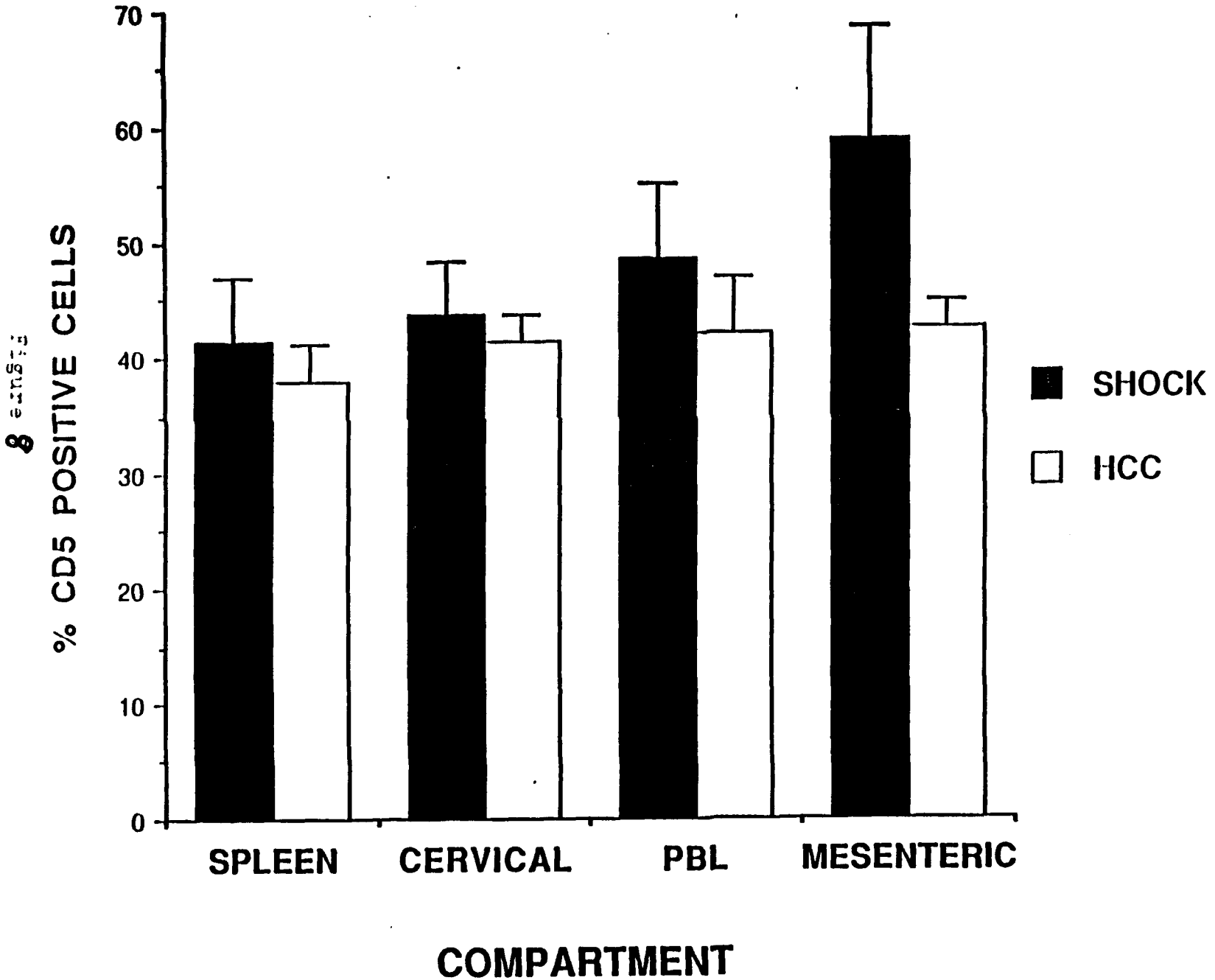


Figure 9

SHOCK-INDUCED INCREASE TREND IN CD5+ MESENTERIC LYMPHOCYTE



NO EFFECT OF SHOCK ON Ig+ LYMPHOCYTES

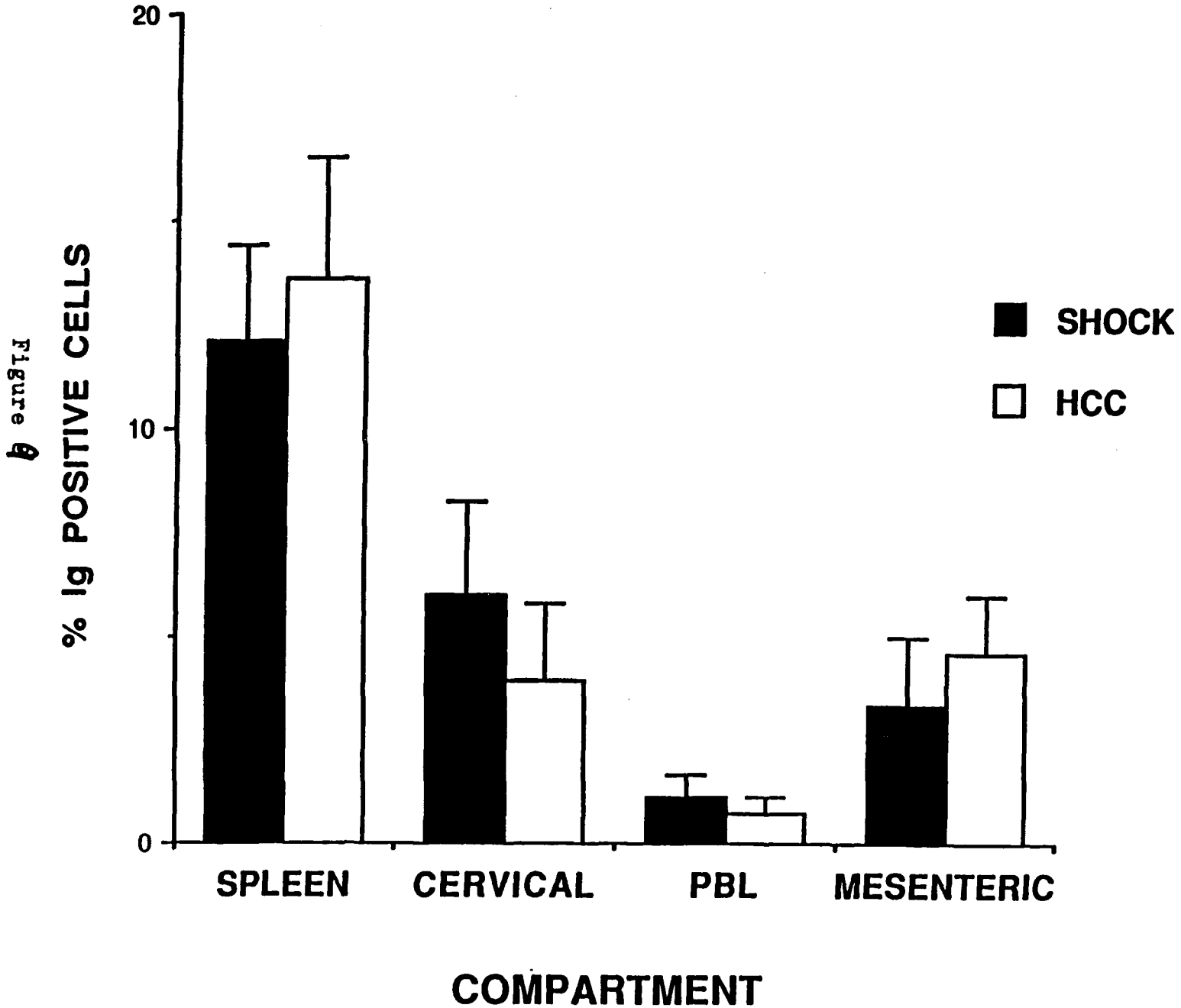


Figure 4

SPLENECTOMY BLOCKS SHOCK-INDUCED RATIO INCREASE OF MESENTERIC LYMPHOCYTES

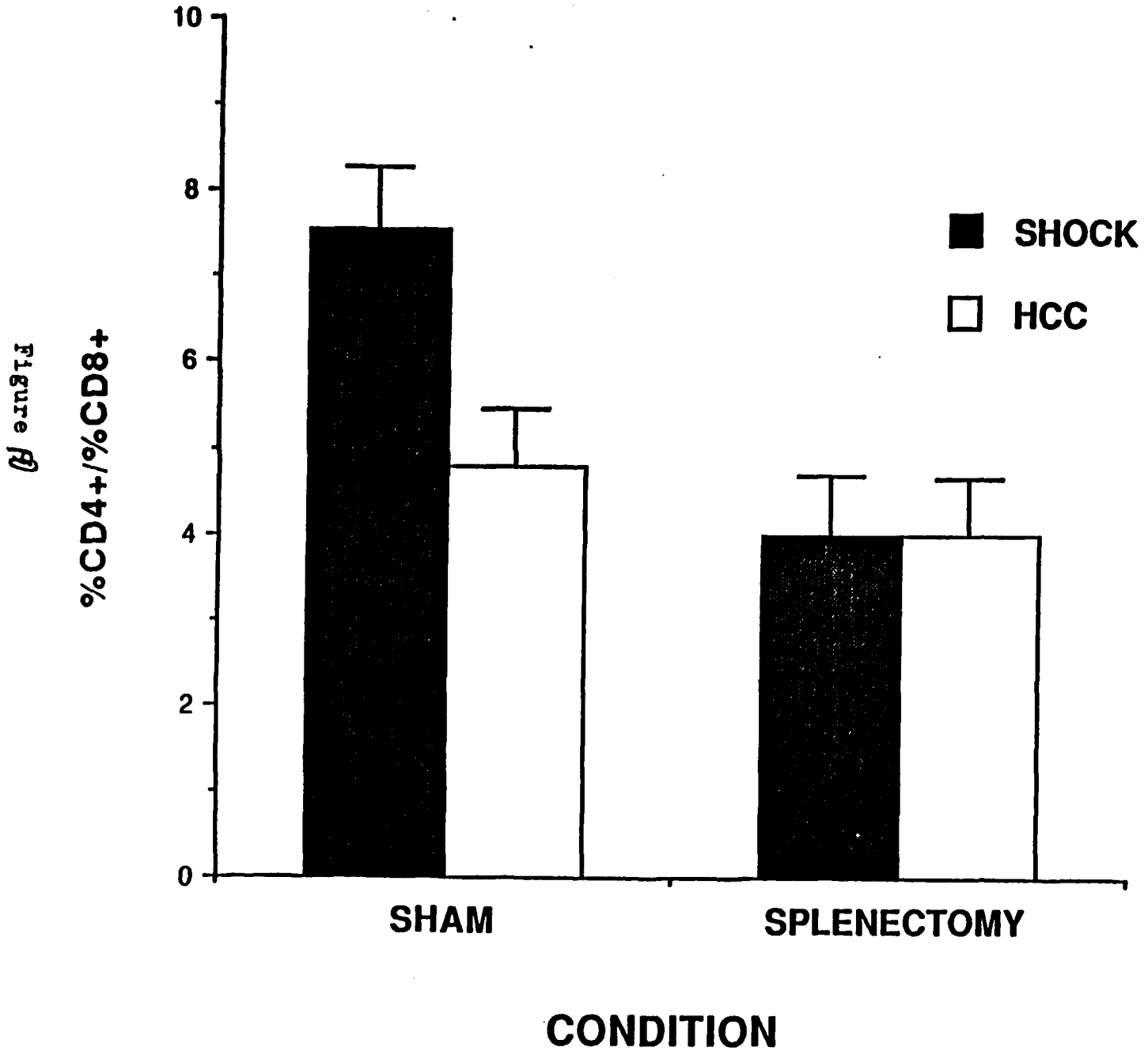
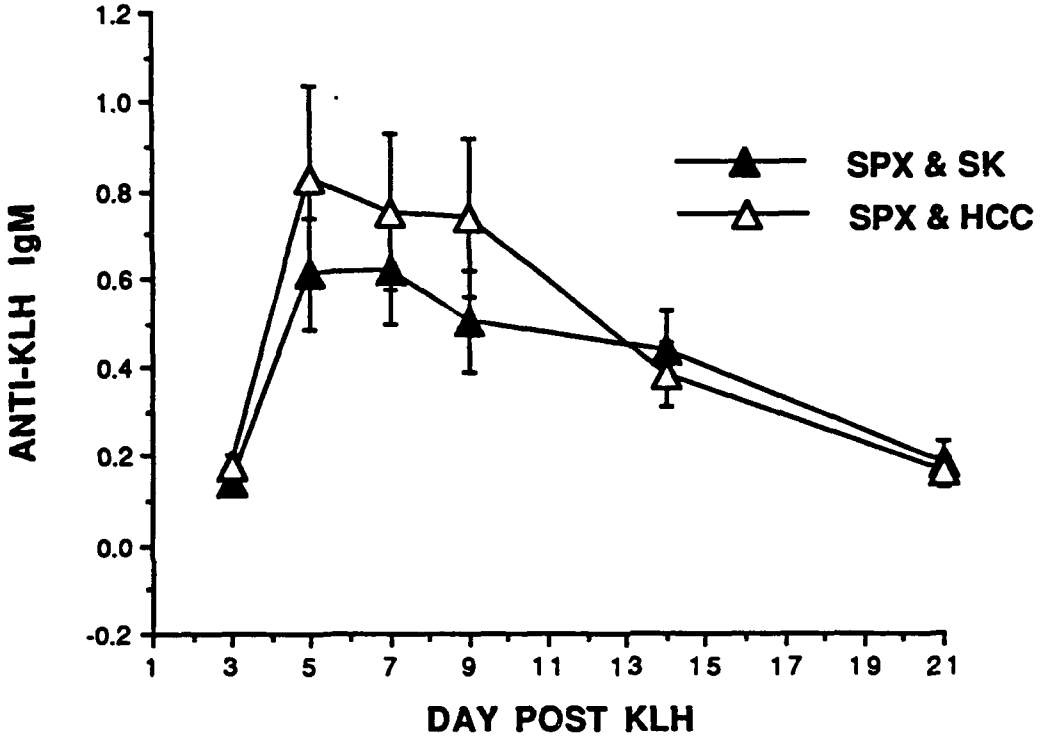


Figure 8

SPLENECTOMY PREVENTS SHOCK-INDUCED SUPPRESSION OF IgM



SPLENECTOMY PREVENTS SHOCK-INDUCED SUPPRESSION OF IgG

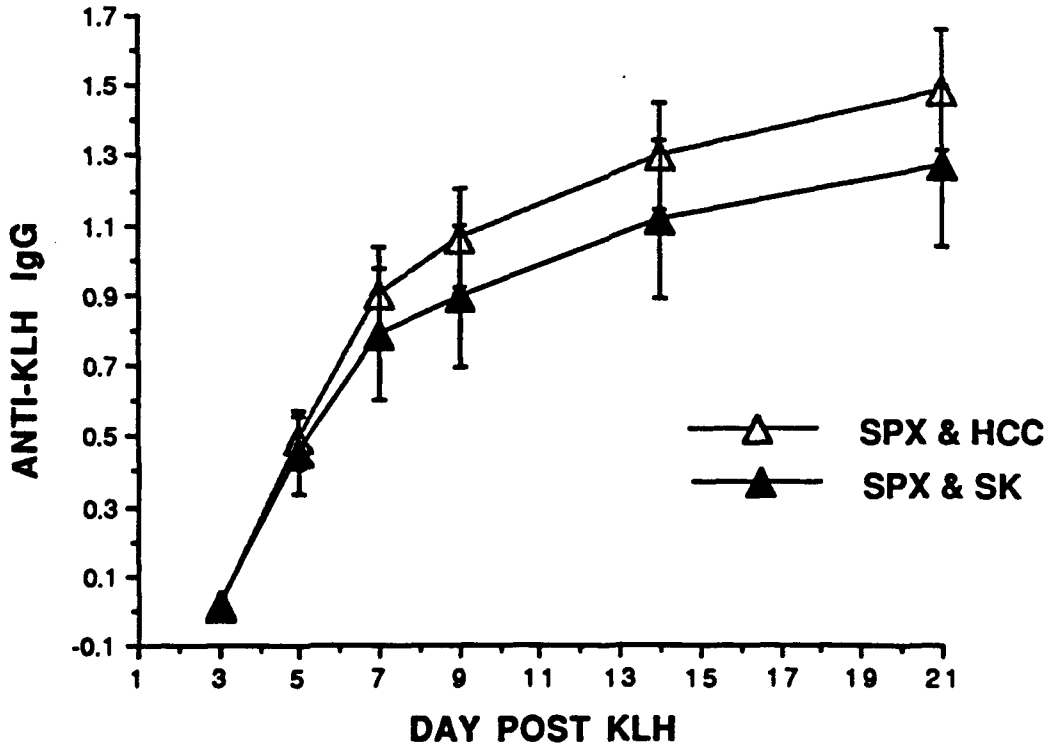


Figure 19

SHOCK INDUCES CD4/CD8 INCREASE IN THE PRESENCE OF KLH

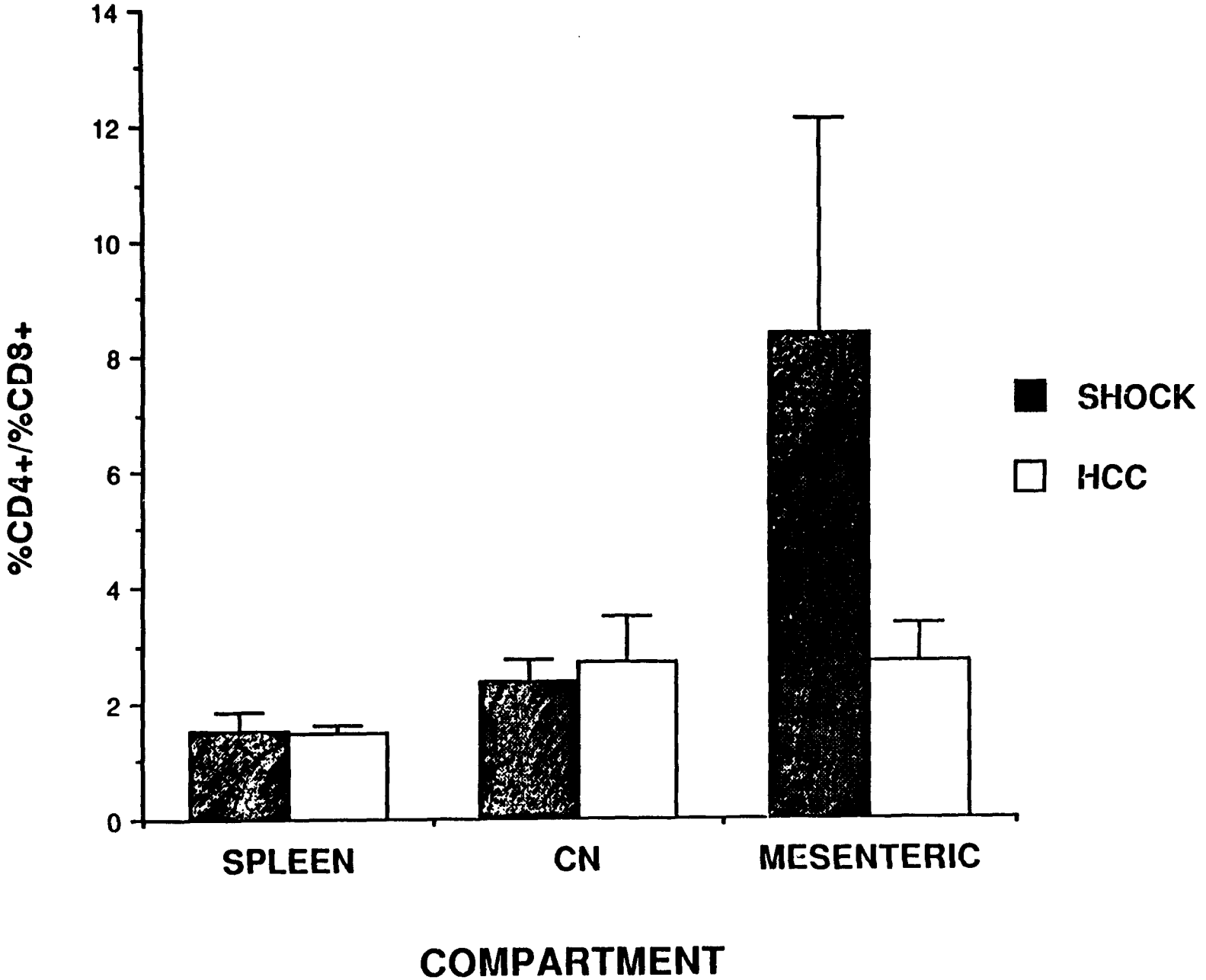


Figure 12

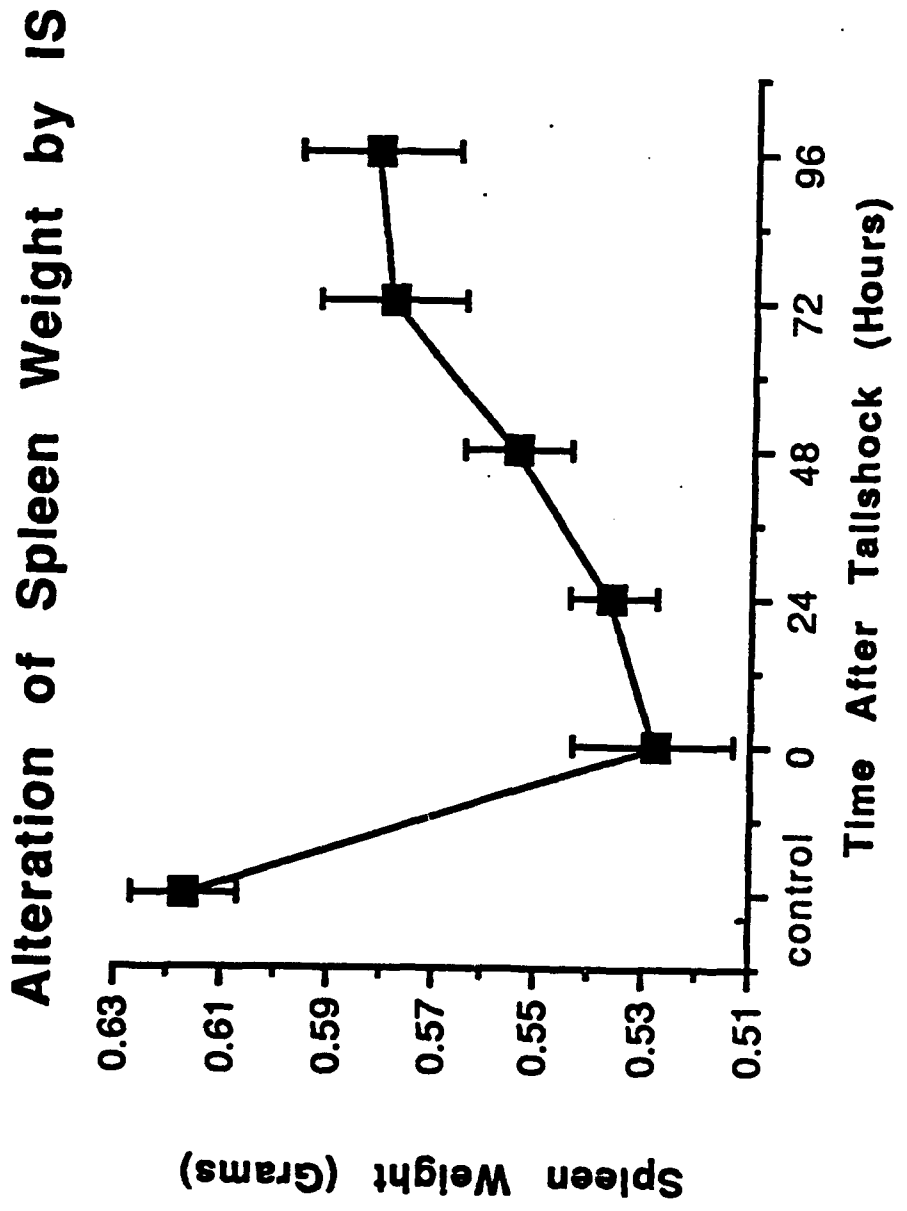

Alteration of Spleen Weight by IS
Spleen Weight (Grams)
0.63
0.61
0.59
0.57
0.55
0.53
0.51
control
0
24
48
72
96
Time After Tailshock (Hours)

Figure 13